

PARENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION
(PCT Rule 61.2)

Date of mailing (day/month/year) 20 February 2001 (20.02.01)	To: Commissioner US Department of Commerce United States Patent and Trademark Office, PCT 2011 South Clark Place Room CP2/5C24 Arlington, VA 22202 ETATS-UNIS D'AMERIQUE in its capacity as elected Office
International application No. PCT/GB00/02254	Applicant's or agent's file reference SJW/8002-PCT
International filing date (day/month/year) 21 June 2000 (21.06.00)	Priority date (day/month/year) 22 June 1999 (22.06.99)
Applicant WORRALL, Eric, Edward	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:

15 January 2001 (15.01.01)

in a notice effecting later election filed with the International Bureau on:

2. The election was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Juan Cruz Telephone No.: (41-22) 338.83.38
---	---

REC'D 07 AUG 2001

WIPO

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SJW/8002-PCT	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/GB00/02254	International filing date (day/month/year) 21/06/2000	Priority date (day/month/year) 22/06/1999	
International Patent Classification (IPC) or national classification and IPC C12N1/04			
Applicant WORRALL, Eric Edward et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 18/01/2001	Date of completion of this report 03.08.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Bretherick, J Telephone No. +49 89 2399 8415



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/02254

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):
Description, pages:

1-16 as originally filed

Claims, No.:

1-26 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
 - the language of publication of the international application (under Rule 48.3(b)).
 - the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:
- contained in the international application in written form.
 - filed together with the international application in computer readable form.
 - furnished subsequently to this Authority in written form.
 - furnished subsequently to this Authority in computer readable form.
 - The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
 - The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
4. The amendments have resulted in the cancellation of:
- the description, pages:
 - the claims, Nos.:
 - the drawings, sheets:
5. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB00/02254

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

1. Statement

Novelty (N) Yes: Claims 1-26
 No: Claims

Inventive step (IS) Yes: Claims 1-26
 No: Claims

Industrial applicability (IA) Yes: Claims 1-26
 No: Claims

2. Citations and explanations
see separate sheet

Regarding Part V : Art. 33(1)(2)(3) EPC:

The current claim set is considered to comprise an inventive step under Art. 33(1)(3) EPC.

The closest prior art is D1, WO 99 27071 A (UNIVERSAL PRESERVATION TECHNOL) 3 June 1999 (1999-06-03), which discloses lyophilised compositions comprising a biologically active material such as a virus, liposome, cell, peptide, protein, antibody coenzyme, a protective substance such as trehalose (page 5, lines 20-24 in order to provide for stability during desiccation and further polypeptides which are designed to increase the glass transition temperature of the mixture during drying, so as to counteract the effect of monosaccharides added in order to prevent crystalisation of the trehalose etc. during desiccation. All biological polymers, including polysaccharides which are highly soluble in concentrated sugar solutions are deemed as suitable, (page 6, lines 3-9).

D2, advocates the use of biopolymers such as chitosan for the imparting viscosity to a reconstituted solution of a lyophilised mixture. In view of the fact that chitosan has a rather poor aqueous solubility, it would not appear to be a good candidate for the methods used in D1 to create lyophilates. It is also used in the current application to provide a protective surface enveloping the biologically active substance, which contrasts with the use in D2. It would therefore not appear logical to combine the information provided in the methods disclosed in D1 with the use of chitosan in D2 and arrive at the subject-matter of the claims of the current application without hindsight.

An inventive step can thus be accorded to the current claims.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference SJW/8002-PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 00/02254	International filing date (day/month/year) 21/06/2000	(Earliest) Priority Date (day/month/year) 22/06/1999
Applicant WORRALL, Eric Edward et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

- the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :
- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. Certain claims were found unsearchable (See Box I).

3. Unity of Invention is lacking (see Box II).

4. With regard to the title,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. —

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02254

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L A01N C12N F26B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA, INSPEC, COMPENDEX, BIOSIS, CHEM ABS Data, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 27071 A (UNIVERSAL PRESERVATION TECHNOL) 3 June 1999 (1999-06-03) abstract page 4, line 34 -page 5, line 35 ---	1-3, 6-15, 17-25
X	US 5 149 653 A (ROSER BRUCE J) 22 September 1992 (1992-09-22) column 2, line 1 - line 32 examples 1,2 ---	1-3, 11-13, 17-25
A	EP 0 308 238 A (ETHICON INC) 22 March 1989 (1989-03-22) page 3, line 19 - line 44 page 4, line 19 - line 25 page 6, line 17 - line 41 -----	1,5-8, 11-13,20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

28 September 2000

Date of mailing of the international search report

06/10/2000

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Menidjel, R

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/02254

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9927071	A	03-06-1999	AU 5459698 A EP 1032647 A		15-06-1999 06-09-2000
US 5149653	A	22-09-1992	AR 240255 A AT 95066 T CA 1333562 A CS 8900402 A DE 68909542 D DE 68909542 T EP 0357709 A ES 2009704 A WO 8906542 A JP 6071423 B JP 2503266 T		30-03-1990 15-10-1993 20-12-1994 15-01-1992 04-11-1993 03-02-1994 14-03-1990 01-10-1989 27-07-1989 14-09-1994 11-10-1990
EP 0308238	A	22-03-1989	AU 2223688 A DK 516788 A GR 88100618 A,B JP 1121223 A NZ 226170 A PT 88538 A ZA 8806943 A		23-03-1989 19-03-1989 22-06-1989 12-05-1989 26-07-1990 31-07-1989 30-05-1990

Neaw
12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/78924 A1

(51) International Patent Classification⁷: C12N 1/04

(21) International Application Number: PCT/GB00/02254

(22) International Filing Date: 21 June 2000 (21.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9914412.3 22 June 1999 (22.06.1999) GB

(71) Applicant and

(72) Inventor: WORRALL, Eric, Edward [GB/GB]; Ty
Mawr, Trefilan, Lampeter SA48 8RD (GB).

(74) Agent: WILKINSON, Stephen, John; Stevens, Hewlett
& Perkins, 1 St. Augustine's Place, Bristol BS1 4UD (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/78924 A1

(54) Title: METHOD FOR THE PRESERVATION OF BIOLOGICALLY-ACTIVE MATERIAL

(57) Abstract: Biologically-active material can be preserved by a method of desiccation, without lyophilisation, in a matrix of glassy trehalose. The method involves forming a coacervate of the biologically-active material and chitosan and then dehydrating mixture of coacervate and trehalose solution. In a cycle time much shorter than a typical freeze drying process biologically-active material, such as viruses, proteins and nucleic acids, can be preserved to provide a material that can be rehydrated. The invention is especially useful for the production of vaccines from preserved material.

METHOD FOR THE PRESERVATION
OF BIOLOGICALLY-ACTIVE MATERIAL

The present invention relates to the preservation of biologically-active material, e.g. viruses, bacteria and biomolecules. In particular, it relates to an ultra-rapid method by which such material can be preserved using chitosan and the disaccharide trehalose. By this method a long term preservation of biomolecules and microorganisms can be achieved and, especially, living attenuated vaccines can be prepared.

The preservation of biodegradable materials by dehydration and osmoconcentration is a familiar and ancient technology. When the task of preserving sensitive biomolecules became necessary, simple drying by dehydration failed, as structural water was removed, causing subsequent denaturation and loss of vital activity. Cryopreservation in liquid nitrogen and lyophilisation have become the accepted methods for the long term preservation of sensitive biomolecules, the latter method being used extensively for the preservation of live attenuated vaccines.

Improved thermotolerance of freeze dried Rinderpest vaccine has been achieved by extending the secondary drying cycle, in order to reduce residual moisture (RM) levels to around 1%-2%. This entails long and high energy consuming operational cycles of up to 72 hours as described by Mariner, J.C. et al., Vet. Microbiol., 1990, 21, 195-209. Vaccines produced by this method are known and are distinguished from the standard vaccine by the name "THERMOVAX".

As mentioned above, these currently used processes are time consuming and involve high energy input. Furthermore, lyophilisation confers only a modest level of thermotolerance in the final product and refrigeration is still required to reduce deterioration during storage. This is a particular problem for live vaccines to be used in tropical climates since these lose potency with the unfortunate result that vaccination programs carried out in the field in tropical countries, where monitoring the "cold chain" is difficult, can ultimately

lead to the vaccination of patients with substandard or, in some cases, useless vaccine.

During evolutionary natural selection, certain species of plants and animals acquired the remarkable and elegant ability to tolerate extreme dehydration, remaining dormant in hostile environments for very long periods of time and yet able to assume complete vital activity on rehydration. Examples include the resurrection plant *Selaginella lepidophylla*, the brine shrimp *Artemia salina* (Clegg, J., J.Comp.Biochem.Physiol, 1967, 20, 801-809), the yeast *Saccharomyces cerevisiae* (Coutinho, E., Journal of Biotechnology, 1988, 7, 23-32) and the tardigrade *Macrobiotus hufelandi* (Kinchin, I.M., Biologist, 1995, 42, 4). Such organisms are termed cryptobiotic and the process by which they survive is known as anhydrobiosis. All species of animals and plants which display this ability contain the disaccharide trehalose (α -D-glucopyranosyl- α -D-glucopyranoside). Its presence, generally in the order of 0.2g/g dry cell weight in most cryptobionts, enables them to resist extreme dehydration, high temperatures, X-rays and also, in some species of tardigrades, pressures as high as 600MPa.

Colaco et al., Biotechnology, 1992, 10, 1007-1011, describe the benefit of rapid drying of biological materials using trehalose. This method mostly refers to the drying of restriction enzymes and immunoglobulins onto preformed solid matrices, such as cellulose fibres or onto the surfaces of plastic plates for diagnostic purposes such as ELISA or similar diagnostic applications in the laboratory. Problems arise, however, with these techniques when scaling up to industrial applications, such as large scale commercial vaccine production where much larger unit numbers and volumes have to be handled using mandatory aseptic techniques in partially sealed vials. To meet the operational requirements of large scale vaccine production, where unit volumes from 1.0ml upwards and production batches of 20 litres are typical, a different strategy is required to remove the volume of water in an economically acceptable time. Drying at atmospheric pressure even at the highest physiologically tolerated temperatures would require an unacceptably

long time to remove water quickly enough from partially stoppered vaccine vials and would inevitably result in denaturation and loss of potency.

Nishimura. K. et al, VACCINE 3 No. 5 (1985) show that chitosan coacervates have a high affinity for mucosal epithelia. It was suggested that this might intensify the active transport of antigen into the cells, thus stimulating both IgA and IgG systemic immune response.

The present invention is primarily concerned with a method of preserving biologically-active material using chitosan and trehalose under conditions which cause water to be removed while, at the same time, allowing the biological integrity of the material to be maintained.

Accordingly, the invention provides a method of preserving biologically-active material which comprises mixing an aqueous suspension of the biologically-active material with a sterile aqueous solution of chitosan or non-toxic salt thereof to form a coacervate of the biologically-active material and chitosan or non-toxic salt thereof, adding to the coacervate a sterile aqueous solution of trehalose, subjecting the sterile aqueous mixture of coacervate and trehalose to drying at a pressure of less than atmospheric and at a temperature, initially no greater than 37°C, which is controlled not to fall to 0°C or below to form a glassy porous matrix comprising metastable glassy trehalose containing, within the matrix, chitosan or non-toxic salt thereof and desiccated biologically-active material.

According to a preferred embodiment, the method of preserving a biologically-active material comprises the steps:

- (i) mixing an aqueous suspension of the biologically-active material with a sterile aqueous solution of chitosan or a non-toxic salt thereof to form a coacervate of the biologically-active material and chitosan or non-toxic salt thereof;
- (ii) mixing with the coacervate produced in step (i) a sterile aqueous solution of trehalose;
- (iii) subjecting the mixture to primary drying, for 30 to 60 minutes, at a pressure of less than atmospheric and at a temperature initially no greater than 37°C, and which is controlled not to fall to 0°C or

below and which finally is no greater than 40°C, to form a glassy porous matrix comprising glassy trehalose having a residual moisture content of not greater than 10% and containing, within the matrix, chitosan or non-toxic salt thereof and desiccated biologically-active material; and

- (iv) subjecting the glassy porous matrix of step (iii) to secondary drying for 10 to 30 hours at a pressure not greater than 0.1 mbar and at a temperature which finally is in the range of from 40 to 45°C to form a trehalose matrix having a residual moisture content of not greater than 2% containing, within the matrix, chitosan or non-toxic salt thereof and desiccated biologically-active material.

By using the method of the invention it is possible to produce a live vaccine with, compared to prior art methods, enhanced biological characteristics and distinct commercial advantages. Vaccines prepared using the method of the invention are dried much more quickly than those using conventional freeze drying procedures. For instance, the method of the invention can be used to prepare trehalose/chitosan/biologically-active material mixtures to a moisture content of about 10% in less than one hour. Further dehydration to a residual moisture content of about 1-2% can be achieved in less than 30 hours, for instance about 20 hours compared to a period of 50 hours by conventional freeze drying procedures. Furthermore, damage caused by solute concentration is minimised according to the present invention and particularly damaging ice crystallisation is avoided. The thermostability of the biologically-active material preserved in the trehalose glassy matrix is greater than that of materials preserved by prior art methods and, thus, the necessity of the "cold chain", which is a serious constraint with conventional freeze-dried vaccine, is minimised. The product of the present invention can be exposed to high ambient temperatures, e.g., up to about 45°C, for prolonged periods without serious loss of biological activity. In addition to these, and other advantages, of the present invention the product of the method exhibits instantaneous "flash solubility" upon rehydration.

The method of the present invention is suitable for achieving the long term preservation of biologically-active materials, for example, microorganisms, proteins, living biomolecules and nucleic acids. In particular, it can be used to preserve highly labile live attenuated viral components and mycoplasma components that can be rehydrated to form vaccines. Examples of such biologically-active materials that can be preserved according to the method of the invention include:

Family: Paramyxoviridae

Subfamily: Paramyxovirinae

Genera: Parainfluenza virus group, Measles, Rinderpest, canine distemper, Peste des Petits Ruminants (PPR)

Paramyxovirus: mumps virus (Mumps)

Genus: Rubivirus, Rubella (German Measles)

Genus: Flavivirus, Yellow fever virus (Yellow Fever)

Genus: Rhabdoviruses, Lyssaviridae (Rabies virus)

Picorna viruses (Polio virus)

Newcastle Disease virus

Mycoplasma: Mycoplasma mycoides (Contagious Bovine Pleuropneumonia)

Brucella abortus: Strain 19 vaccine.

According to the method of the present invention, the biologically-active material to be preserved is prepared as a suspension in an appropriate aqueous medium. It may be the case that a virus or bacterial strain will need to be cultured, for instance in vitro cells, in an appropriate culture medium, and then harvested prior to suspension in order to provide a useful concentration of material. Typically, the aqueous suspension of biologically active material will be pH adjusted, for example by the addition of an alkali, to a pH in the range of from 7.0 to 7.8 especially about 7.4.

The suspension of biologically-active material will then be mixed with a sterile aqueous solution of chitosan or a non-toxic salt thereof. Chitosan is the general name given to a class of cationic polysaccharides prepared by the deacetylation of chitin, (poly N-acetyl glucosamine) to (poly,1,4-beta-D-

glucopyranosamine). Chitin is a natural biopolymer which occurs abundantly in the exoskeletons of crustaceans. The cuticles of tardigrades in the highly resistant "tun" state are composed of chitin. Chitosan is a linear cationic polyelectrolyte which is non-toxic, biodegradable and biocompatible and is a white or off-white amorphous translucent solid which is soluble in dilute organic acids. The use of chitosan in the present invention is based on the discovery that it acts as a protective biopolymer shell around the biologically-active material being preserved. Thus, the use of chitosan in combination with the controlled method of desiccation described herein improves the thermal tolerance of the desiccated material. Typically, the concentration of the chitosan in the sterile aqueous solution used for admixture with the suspension of biologically-active material will be in the range of from 0.001% to 0.02% w/v. Excellent results have been obtained using a solution containing 0.01% w/v chitosan. The chitosan is preferably used in the form of a non-toxic acid addition salt, e.g., chitosan HCl, which is more soluble than chitosan.

The chitosan solution and the suspension of biologically-active material are typically mixed in a volume ratio of about 1:1, at a pH of 7.4, at a temperature of from above 0°C to 37°C, preferably less than 20°C and most preferably about 4°C. The chitosan and the biologically-active material are preferably subjected to vigorous agitation, e.g., by vortex stirring for up to 60 seconds at a temperature in the range of from 2 to 6°C, preferably about 4°C to produce a homogeneous mixture. The mixture will, typically, be allowed to stand while maintaining the temperature to allow a coacervate, i.e. an absorption complex of the chitosan and the biologically-active material wherein the biologically-active material becomes coated with the chitosan, to form. The thus-formed coacervate is then collected, typically by centrifugation. This chitosan coacervate precipitation is useful for concentrating low titre vaccine pools and/or for reducing process volumes for drying. Preferably, the centrifugation step is carried out not more than one hour after mixing the chitosan solution and the suspension of biologically-active material. If the mixture is stored for a longer period the particles of

coacervate precipitate, rather than being light and homogeneous, tend to clump together. The centrifugation is preferably conducted in a refrigerated centrifuge, typically maintained at about 4°C, at a typical speed of about 10,000 rpm for several minutes. Following collection, the coacervate is preferably resuspended in an appropriate medium, e.g. Hanks balanced salt solution (HBSS) or Hanks lactalbumin yeast extract (Hanks LYE) before being mixed with a sterile solution of trehalose. The coacervate will, in general, be resuspended in a volume of appropriate medium which is equal to the volume of the liquid prior to centrifugation so that the concentration of biologically-active material in the coacervate suspension is the same as it was in the liquid prior to centrifugation. However, the biologically-active material can be concentrated, if desired, by resuspending the coacervate into a volume of appropriate medium which is smaller than the volume of the liquid prior to centrifugation. By concentrating the biologically-active material in this way the volume of water that needs to be removed during the vacuum drying stage is effectively reduced, thus reducing the amount of energy required during evaporative drying and reducing the period of time required to dry the material in accordance with the method of the invention. In the case where the biologically-active material is a virus it may often be beneficial to concentrate the virus, i.e., to increase the titre of the virus; in this way.

Trehalose is one of the most stable and chemically non-reactive disaccharides. It has an extremely low bond energy of less than 1Kcal/Mol making the dimer structure very stable. It does not undergo caramelisation unless heated severely, nor does it cause the Maillard reaction with proteins or peptides. The natural di-hydrate structure containing two water molecules enables unusual flexibility around the disaccharide bond which possibly permits a closer association with tertiary structured biomolecules. It is not hygroscopic yet exhibits "flash solubility" on hydration, a property particularly useful for dried vaccines.

The sterile aqueous solution of trehalose used in the method of the present invention will typically have a trehalose concentration of from 0.20% to 10% w/v, although it is possible to use solutions having a higher

concentration (e.g., 50% w/v) of trehalose to provide the final required concentration of trehalose in the mixture which is subjected to drying. Preferably, the trehalose concentration will be 2 to 10% and more preferably 2.5 to 8% w/v. Within the range of trehalose concentrations, the actual concentration used will, in general, depend on the unit size of the biologically-active material. Less trehalose is required for small virus particles than for large cells.

The mixture of the sterile aqueous solution of trehalose and resuspended coacervate, when prepared, is subjected to vacuum drying. Preferably a conventional freeze drying apparatus (e.g., such as manufactured by EDWARDS, CHRIST, USIFROID or SAVANT) is used for the drying procedure in order to provide a controlled environment for the critical stages in the method. It is emphasised, however, that if such an apparatus is used freeze drying conditions are not employed and the material is not subjected to freeze drying. The initial temperature of the drying apparatus is such as to ensure that the trehalose/coacervate mixture will not be greater than 37°C and will preferably be 37°C in order to prevent any loss of biological activity at this stage in the method. As water is evaporated off the mixture, the temperature of the mixture falls. The desiccation is, however, carried out to ensure that no freezing or sublimation from ice occurs as is normally experienced in conventional freeze drying procedures, i.e. the temperature of the product during this stage of the drying process is controlled not to fall to 0°C or below and is typically controlled not to fall below 4°C. The pressure is reduced below atmospheric pressure, preferably to a value of from 800 to 300 mbar.

The drying stage is continued for a period of time, typically between 30 and 60 minutes, during which time the temperature of the trehalose/coacervate mixture initially falls as water is evaporated off and then rises. When a temperature (under the reduced pressure employed) of about 25°C is reached the trehalose forms a glassy matrix. The moisture content of the glassy matrix is about 10%. The temperature of the material at the end of this drying procedure should preferably be controlled so that it does not

exceed 40°C since the biologically-active material will sustain damage if the temperature is allowed to rise above this.

It is preferred that the method of the invention utilizes a drying procedure that comprises two drying stages, a primary drying stage (as described above) in which the residual moisture content of the material is reduced to a value of not greater than 10% and a secondary drying stage in which the residual moisture content of the material is reduced further to a value not exceeding 2%. The secondary drying stage, if used, is carried out following a primary drying stage as described above preferably without removal of the material from the drying apparatus used for the primary drying stage. In a secondary drying stage the glassy matrix obtained at the end of the primary drying stage is further dehydrated under a reduced pressure not exceeding 0.1mbar. Currently-available specialised apparatus has the ability to operate at extremely low pressures, for instance below 0.001 mbar. However, very good results have been obtained according to the present invention using reduced pressures for the secondary drying stage in the range of from 0.001 to 0.1mbar, typically from 0.01 to 0.1 mbar. The secondary drying stage is continued for a total period of time in the range of from 10 to 30 hours, preferably 20 to 30 hours.

As mentioned above, the temperature of the glassy matrix at the end of the primary drying stage should not exceed 40°C. During the secondary drying stage the temperature of the matrix may rise slightly to a final temperature, at the end of the drying procedure, of from 40°C to 45°C. The trehalose matrix, at the end of the secondary drying stage will have a residual moisture content of 2% or less, preferably 1% or less, in order to ensure a very high degree of thermostability in the product. At such residual moisture contents the final temperature in the drying process should not be higher than 45°C since the desiccated biologically-active material in the matrix may undergo some degree of destruction above 45°C. Preferably, the temperature of the product at the end of the secondary drying stage will not exceed 44°C.

It has been found, through experimentation that there is some benefit in controlling the temperature of the matrix, during the secondary drying stage, such that it is maintained at about 37°C for a period of from 15 to 17 hours and then raising this gradually (for instance by 0.5 to 1°C per hour), over the remaining secondary drying time to a final temperature within the range of 40°C to 45°C.

The glassy trehalose matrix produced according to the method can be stored for long periods of time, e.g. several weeks, without any substantial loss of vital activity and without the need for refrigeration and can be rehydrated very quickly in an appropriate aqueous medium, typically sterile distilled water, to produce a vaccine for use in a very short period of time.

The preparation of a vaccine and the operating procedure using a freeze dryer for the desiccation of biologically-active material such as the viruses Rinderpest and Peste des Petits Ruminants viruses, and mycoplasma without a lyophilisation step involving sublimination from ice, exploits the unique property of the disaccharide trehalose to protect tertiary macromolecules during desiccation.

Compared to conventional freeze-drying procedures the method of the invention offers the following benefits:

- It provides a high level of protection for the biologically-active material, employing a relatively short, simple procedure. An even higher level of protection is provided by using the preferred two stage drying procedure which may use a 25 hour cycle.
- Production cycle time and energy costs are, thus, reduced compared to typical freeze drying procedures.
- Basic drying equipment is all that is required, although sophisticated microprocessor controlled freeze dryers can also be used, but are not strictly essential.
- The method is tolerant of power interruption, unlike lyophilisation where even a short power failure can cause product melting, leading to unacceptable loss of virus.

Oral vaccination with some attenuated strains of virus has in the past been difficult to achieve because of the loss of epitheliotropism. Both oral and intranasal vaccination would be useful and appropriate for many applications because they mimic the natural route of droplet infection, generating a cascade of protective mucosal immunity, with IgA and humoral IgG2a T helper-cell type 1 response. It would be easy to administer such routes of vaccination and these would be applicable in the event that a suitable vaccine is prepared. Chitosan increases the transcellular and paracellular transport across mucosal epithelium and is thought to enhance or restore diminished epitheliotropism commonly associated with attenuated vaccine strains (e.g., attenuated RBOK Rinderpest virus and CBPP S1/44 mycoplasma). This may be particularly important in CBPP and other mycoplasma infections where the protective mechanisms are still obscure, particularly post vaccination immunity following sub-cutaneous vaccination. A vaccination procedure with live attenuated strains mimicking the natural route of infection induces a more comprehensive sero mucous and cell mediated immunity. According to a further aspect the present invention provides a method of making a vaccine for oral or intranasal use which comprises preparing a glassy matrix of trehalose containing desiccated virus according to the above described method and rehydrating the glassy matrix with an appropriate aqueous composition. According to a preferred embodiment of this aspect of the invention the vaccine for oral or intranasal vaccination is an MMR vaccine. Since the current paediatric MMR vaccine is prepared by conventional freeze drying technology and is injected into the patient subcutaneously, an oral or intranasal vaccine would give great benefits.

EXAMPLE 1

Method for the preparation of a bivalent live attenuated veterinary vaccine

Rinderpest virus RBOK strain was grown in vero cells in Hanks LYE (lactalbumin hydrolysate and yeast extract) medium containing 0.1% trehalose instead of glucose. Contagious Bovine Pleuropneumonia (CBPP) *Mycoplasma mycoides subs.mycoides* S1/44 (SC) T₁-SR was grown in

Gourlay medium. The virus pool and the CBPP pool were then harvested. The pH of the virus and the CBPP pool were adjusted with 0.1M NaOH to pH 7.4.

A stock 2% w/v solution of chitosan was prepared as follows:

Chitosan HCl (supplied by Pronova Seacure)	20g
Distilled water	1000ml

Autoclaved at 121°C for 30 minutes.

A stock solution of 50% w/v trehalose dihydrate in Hanks Balanced Salt Solution (HBSS) was prepared the pH being adjusted by the addition of 0.1M NaOH to 7.4. The solution was sterilised by autoclaving at 121°C for 20 minutes.

A suitable volume of working strength 0.02% w/v chitosan was prepared by adding 1ml of stock 2% w/v chitosan to 99ml of sterile distilled water.

One volume of 0.02% w/v chitosan solution was added to one volume of virus fluid at 4°C and the pH adjusted to 7.4 with 0.1M NaOH. This step was repeated separately for the CBPP culture pool and the resulting coacervation complex of each was completed by rapid vortex stirring for 30 seconds and subsequently stored at 4°C for 1 hour. The resulting precipitate of each was collected by centrifugation at 10,000 rpm in a refrigerated centrifuge, the supernatant discarded and the coacervate resuspended in one volume of Hanks balanced salt solution (HBSS). A volume of sterile 50% w/v solution of trehalose in Hanks balanced salt solution (HBSS) was added to the coacervate suspension give a final trehalose concentration of 5% w/v (potency is checked by the quality control standard operating procedures for these organisms).

The vaccine was dried by filling 1.0ml aliquots into 5ml vaccine vials partially stoppered with dry butyl rubber stoppers. The shelves of the conventional freeze dryer (EDWARDS MODULYO) were heated to 37°C and the condenser was allowed to reach minus 40°C. The vaccine vials were placed in the dryer and the pressure in the drying chamber was adjusted to 800 mbar and drying commenced for 30 minutes until approximately 75% of the water had evaporated, taking care not to allow the product temperature to

fall below 0°C. The pressure was then lowered to 500 mbar and drying was continued until the glass transition temperature of trehalose was reached at approximately 25°C, a glassy porous matrix was formed, and the temperature of the product was allowed to rise to reach the initial starting temperature close to that of the shelves. At this stage the residual moisture (RM) was approximately 10%. Further drying at 0.01 mbar and 45°C for 17 hours reduced this to 1-2% RM ensuring high thermostability in the product.

The pressure was maintained at 0.01 mbar and the vials were sealed under vacuum or at atmospheric pressure under dry nitrogen.

Results

The effectiveness of the present invention was demonstrated by measuring the titres of Rinderpest virus and the CBPP mycoplasma in their respective parent liquids and then in vaccines prepared from the dehydrated product obtained according to the Example above.

Rinderpest Virus

1. Virus titre of parent liquid before drying 5.40 Log 10 TCID 50/ml.
2. Virus titre in product of the Example (dried with 2.5% trehalose) 5.45 Log 10 TCID 50/ml.

where TCID 50/ml = tissue culture infective dose 50 per ml.

CBPP mycoplasma

1. Titre of live mycoplasma parent liquid before drying 9.90 Log 10 orgs/ml.
2. Titre of product of the Example (after drying with 5% trehalose) 8.05 Log 10 orgs/ml

EXAMPLE 2

Materials and Methods

Preparation of a PPR vaccine culture

Peste des petits ruminants (PPRV 75/1) strain was grown initially using vero cells in Glasgow modication Eagles medium (GMEM) supplemented with 10% tryptose phosphate broth (TPB, Difco) and 10% foetal calf serum (FCS) as follows:

Vero cells were seeded into 5 x 150cm² plastic flasks at a cell concentration of 287,000 cells/ml, 60ml per flask. Two flasks were inoculated with 0.5ml PPR virus suspension at a multiplicity of infection of 0.03 virus particles/cell. One flask remained uninoculated as a control.

The flasks were incubated at 37°C in 5% CO₂ and cells examined daily for development of cytopathic effects (cpe). On day 4 the GMEM medium was replaced with Hanks lactalbumin yeast extract (Hanks LYE), containing 2% FCS and 0.1% trehalose dihydrate. On day 6 the cpe was approximately 80% and the virus harvests were pooled, frozen and stored at -20°C. The control flask remained in the incubator for 10 days, and proved to be free from contamination or cell degradation, with no obvious sign of adventitious agents.

Chitosan Solution

A stock 2% w/v aqueous solution of chitosan (Seacure CI 310) was prepared in distilled water and sterilized in an autoclave at a temperature of 121°C for 30 minutes. The stock solution was diluted using sterile distilled water to give a solution containing 0.02% w/v chitosan.

Method

The pH of the virus fluid was adjusted to 7.4 using 0.1 M NaOH. To one part of the pH adjusted virus fluid was added one part of the 0.02% w/v diluted chitosan solution and the mixture was vortex stirred for 30 seconds. The stirred mixture was allowed to rest for 1 hour at 4°C to allow complete formation of the coacervate complex.

Immediately, at the end of the one hour rest period, the coacervate mixture was centrifuged at 10,000 rpm in a refrigerated centrifuge for 20 minutes. The resulting supernatant was discarded and the precipitated complex was collected and suspended in Hanks LYH medium +2% FBS at a pH 7.4. The total volume of the resuspension medium was equal to the total volume of the liquid as submitted to centrifugation.

One part of the resuspended complex was mixed with one part of a sterile 5% w/v aqueous solution of trehalose dihydrate, thus giving a fluid trehalose concentration of 2.5% w/v trehalose in the mixture.

One ml volumes were distributed into each of 5ml vaccine vials and partially sealed with dry vented butyl rubber inserts. This application was carried out at room temperature, in a laminar air flow biohazard cabinet and observing strict aseptic precautions.

Primary Drying

The dehydration process was carried out using an Edwards Supermodulyo freeze dryer with precise control over chamber pressure, condenser pressure, shelf and product temperatures. The freeze dryer was prepared in advance before loading the shelf chamber with the vials containing the product. The shelf temperature was raised to 40°C and the condenser temperature was allowed to reach the operational limit of -40°C. Vials were then placed on the shelves and the contents allowed to reach 35°C. The chamber door was closed with the macro and micro air admittance valves fully opened and the vacuum pump switched on with full gas ballast. The pressure in the chamber was adjusted to 800mbar by carefully closing the macro air admittance valve. The pressure in the condenser was maintained at 500mbar in order to produce a pressure gradient between the chamber and condenser and this provided the driving force to induce water vapour to flow from the product surface to the condenser. Partial closure of the vials with the stoppers also had the beneficial effect of throttling the aperture thus increasing the pressure still further at the product surface. It was noticed that the partially closed vials dried quicker than the fully open ones containing the temperature recording thermocouples.

The product temperature was controlled primarily by carefully closing the macro air admittance valve during the first 15 minutes, and thereafter by manipulation of the micro air admittance valve, making sure not to allow the product to freeze. Maintaining a temperature around 1-2°C caused by evaporative cooling, increased the evaporation rate so that 90% of the water had evaporated within one hour and the product temperature began to rise to match the shelf temperature. As dehydration proceeded a critical point was reached after 40 minutes when there was a sudden rapid rise to 25°C

followed within seconds by a sudden fall in product temperature to 15°C. This was accompanied by a dramatic bubbling of the product.

Secondary Drying

The batch was then subjected to a period of secondary drying where the temperature was raised over a period of a further 17 hours to a final product temperature of 42.4°C and a pressure of 0.06mbar, with gas ballast fully closed. This had the effect of reducing the residual moisture content of the material to approximately 0.72%.

CLAIMS

1. A method of preserving biologically-active material comprising mixing an aqueous suspension of the biologically-active material with a sterile aqueous solution of chitosan or a non-toxic salt thereof to form a coacervate of the biologically-active material and chitosan or non-toxic salt thereof, adding to the coacervate a sterile aqueous solution of trehalose, subjecting the sterile mixture of coacervate and trehalose to drying at a pressure less than atmospheric and at a temperature, initially no greater than 37°C, which is subsequently controlled not to fall to 0°C or below to form a glassy porous matrix comprising metastable glassy trehalose containing, within the matrix, desiccated biologically-active material and chitosan or non-toxic salt thereof.
2. A method according to claim 1, wherein the biologically-active material is selected from viruses, bacteria, tertiary structured biologically-active protein and nucleic acid.
3. A method according to claim 2, wherein the biologically-active material is at least one virus selected from Rinderpest virus, Peste de Petit Ruminants virus, Measles, Mumps, Rubella, Yellow Fever, Polio and Newcastle Disease Virus.
4. A method according to claim 2, wherein the biologically-active material is Contagious Bovine Pleuropneumonia (CBPP) mycoplasma.
5. A method according to any one of claims 1 to 4, wherein the sterile aqueous solution of chitosan or non-toxic salt thereof has a chitosan concentration of 0.01% w/v.

6. A method according to claim 5, wherein the sterile aqueous chitosan solution and the aqueous suspension of biologically-active material are mixed at a volume ratio of 1:1 at pH 7.4.
7. A method according to any one of claims 1 to 6, wherein the coacervate of biologically-active material and chitosan is subjected to vortex mixing.
8. A method according to any one of claims 1 to 7, wherein the coacervate of biologically-active material and chitosan is mixed with a sterile aqueous trehalose solution having a trehalose concentration in the range of from 0.20 to 20% w/v.
9. A method according to claim 8, wherein the sterile aqueous solution of trehalose has a trehalose concentration in the range of from 2.5 to 8% w/v.
10. A method according to claim 9 wherein the sterile aqueous solution of trehalose has a trehalose concentration of about 5% w/v.
11. A method according to any one of claims 1 to 10, wherein the mixture of coacervate and trehalose is subjected to drying for 30 to 60 minutes, at a pressure of less than atmospheric and at a temperature initially no greater than 37°C, and which is controlled not to fall to 0°C or below and which is finally no greater than 40°C to form a glassy porous matrix comprising glassy trehalose having a residual moisture content not greater than 10% and containing, within the matrix, desiccated biologically-active material and chitosan or non-toxic salt thereof.
12. A method according to any one of claims 1 to 11, wherein the drying stage is carried out at a pressure of not greater than 800 mbar.

13. A method according to any one of claims 1 to 12, wherein the resulting trehalose matrix containing desiccated biologically-active material and chitosan or non-toxic salt thereof is subjected to a secondary drying procedure for 10 to 30 hours of a pressure not greater than 0.1 mbar and at a temperature which finally is in the range of from 40 to 45°C to form a trehalose matrix having a residual moisture content of not greater than 2% containing, within the matrix, desiccated biologically-active material and chitosan or non-toxic salt thereof.
14. A method according to claim 12, wherein secondary drying is carried out for 20 to 30 hours.
15. A method according to claim 13, wherein secondary drying is carried out for 15 to 17 hours at a temperature of about 37°C and the temperature is, thereafter, raised gradually over the remaining secondary drying time to a final temperature in the range of from 40 to 45°C.
16. A method according to any one of the claims 12 to 14, wherein the residual moisture content at the end of the secondary drying step is 1.0% or lower.
17. A method of making a vaccine comprising preserving a biologically-active material according to the method of claims 1 to 15 and rehydrating the glassy product obtained thereby in an appropriate aqueous medium.
18. A method according to claim 16, wherein the vaccine is for oral or intranasal use.
19. A method according to claim 17, wherein the vaccine is a Measles, Mumps, Rubella (MMR) vaccine.

20. A rehydratable composition comprising trehalose in the form of a metastable glass matrix containing, within the matrix, desiccated biologically-active material and chitosan or a non-toxic salt thereof.
21. A rehydratable composition according to claim 19 which has a residual moisture content of not greater than 2%.
22. A rehydratable composition according to claim 20 which has a residual moisture content of not greater than 1%.
23. A rehydratable composition according to any one of claims 19 to 21, useful on rehydration for making a vaccine.
24. A rehydratable composition according to any one of claims 19 to 22, wherein the biologically-active material is selected from viruses, bacteria, tertiary structured biologically-active proteins and nucleic acids.
25. A rehydratable composition according to claim 23, wherein the biologically-active material is at least one virus selected from Rinderpest Virus, Peste de Petit Ruminants Virus, Measles, Mumps, Rubella, Yellow Fever, Polio Myelitis and Newcastle Disease Virus.
26. A rehydratable composition according to claim 23, wherein the biologically-active material is Contagious Bovine Pleuropneumonia (CBPP) mycoplasma.

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/GB 00/02254A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L A01N C12N F26B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA, INSPEC, COMPENDEX, BIOSIS, CHEM ABS Data, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 27071 A (UNIVERSAL PRESERVATION TECHNOL) 3 June 1999 (1999-06-03) abstract page 4, line 34 -page 5, line 35	1-3, 6-15, 17-25
X	US 5 149 653 A (ROSER BRUCE J) 22 September 1992 (1992-09-22) column 2, line 1 - line 32 examples 1,2	1-3, 11-13, 17-25
A	EP 0 308 238 A (ETHICON INC) 22 March 1989 (1989-03-22) page 3, line 19 - line 44 page 4, line 19 - line 25 page 6, line 17 - line 41	1,5-8, 11-13,20

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

28 September 2000

06/10/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Menidjel, R

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No

PCT/GB 00/02254

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9927071	A	03-06-1999	AU 5459698 A		15-06-1999
			EP 1032647 A		06-09-2000
US 5149653	A	22-09-1992	AR 240255 A		30-03-1990
			AT 95066 T		15-10-1993
			CA 1333562 A		20-12-1994
			CS 8900402 A		15-01-1992
			DE 68909542 D		04-11-1993
			DE 68909542 T		03-02-1994
			EP 0357709 A		14-03-1990
			ES 2009704 A		01-10-1989
			WO 8906542 A		27-07-1989
			JP 6071423 B		14-09-1994
			JP 2503266 T		11-10-1990
EP 0308238	A	22-03-1989	AU 2223688 A		23-03-1989
			DK 516788 A		19-03-1989
			GR 88100618 A, B		22-06-1989
			JP 1121223 A		12-05-1989
			NZ 226170 A		26-07-1990
			PT 88538 A		31-07-1989
			ZA 8806943 A		30-05-1990